AD_____

Award Number: W81XWH-04-C-0071

TITLE: Neuroimmune Effects of Inhaling Low Dose Sarin

PRINCIPAL INVESTIGATOR: Mohan L. Sopori, Ph.D.

CONTRACTING ORGANIZATION: Lovelace Respiratory Research Institute
                            Albuquerque, New Mexico   87108-5127

REPORT DATE: February 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                Fort Detrick, Maryland   21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE February 2005	3. REPORT TYPE AND DATES COVERED Annual (15 Jan 2004 – 14 Jan 2005)

4. TITLE AND SUBTITLE
Neuroimmune Effects of Inhaling Low Dose Sarin

5. FUNDING NUMBERS
W81XWH-04-C-0071

6. AUTHOR(S)
Mohan L. Sopori, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Lovelace Respiratory Research Institute
Albuquerque, New Mexico 87108-5127

E-Mail: msopori@lrri.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The central nervous system (CNS) and the immune system communicate bidirectionally, and cholinergic agents modulate the immune system. Organophosphates, such as the nerve gas sarin, are powerful irreversible inhibitors of ChE, leading to neurotoxicity, seizures, and death. Because of the ease and low cost of production, sarin gas is a tool of mass destruction in the hands of terrorist groups and rogue nations. While people in the immediate vicinity of sarin attack may receive neurotoxic doses, people away from this area are likely to receive subclinical exposures. Even subclinical doses of sarin cause subtle changes in the brain; subclinical exposure to sarin has been proposed as an etiology to the Gulf War Syndrome. Our preliminary experiments suggest that low doses of sarin are highly immunosuppressive, and suppress glucocorticoid production. The effects of sarin exposure on the immune system are attenuated by ganglionic blockers and decreased glucocorticoid level may be a biomarker for cholinergic toxicity. Future experiments are designed to understand the mechanism of sarin-induced immunotoxicity. The study may identify novel biomarkers of nerve gas exposure, and suggest therapeutics to treat the immunotoxicity.

14. SUBJECT TERMS
Sarin, neuroimmune interaction, cholinergic toxicity

15. NUMBER OF PAGES
20

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Table of Contents

INTRODUCTION: The central nervous system (CNS) and the immune system communicate bidirectionally, and cholinergic agents modulate the immune system. Organophosphates, such as the nerve gas sarin, are powerful irreversible inhibitors of cholinesterases, and might cause neurotoxicity, seizures, and death. Because of the ease and low cost of production, sarin gas is a tool of mass destruction in the hands of terrorist groups and rogue nations. Even a subclinical dose of sarin causes subtle changes in the brain. Increasing evidence suggests that the major health effects of sarin are primarily through its effects on the CNS. Although unproven, subclinical exposure of Gulf War veterans to sarin has been implicated in the development of the Gulf War syndrome (GWS). Interestingly, the symptoms of GWS are similar to diseases that result from impaired immune/inflammatory responses and include muscle fatigue, general malaise, myalgia, impaired cognition, ataxia, headaches, fever, joint pain, skin rash, gastrointestinal and sleep disturbances, and respiratory difficulties. Previous studies from our lab showed that repeated exposure to low doses of sarin (0.2-0.4 mg/m^3) for 5 days suppressed T cell proliferation to mitogens and antigens, and inhibited the T cell antigen receptor (TCR)-induced rise in intracellular Ca^{2+} concentration. In addition, sarin dramatically decreased serum corticosterone (CORT) levels. Moreover, effects of sarin on T cell proliferation were blocked by the ganglionic blocker, chlorisondamine. These results suggested that the effects of sarin on T cell responsiveness are mediated through the autonomic nervous system (ANS). In order to understand the mechanism of cholinergic immunotoxicity in general, and of sarin in particular, the following tasks were proposed in the grant application:

Task 1.   To determine whether cholinergic agents require access to the CNS to alter the immune response and corticosterone levels (Months 1-18).
       a.   Ascertain whether cholinergic agents (pyridostigmine bromide, physostigmine, sarin, edrophonium) cause immunosuppression, and whether the development of immunosuppression requires access to the CNS (1-4 months).
       b.   Determine whether cholinergic agents depress corticosterone levels; if so, if it is blocked by ganglionic blockers (5-11 months).
       c.   Establish the kinetics of corticosterone suppression by sarin (12-18 months).

Task 2.   Determine the steps in the antigen-induced T cell signaling pathway that are affected by low-dose sarin inhalation(19-29 months).
       a.   Determine the effects of sarin on Src-like pretein tyrosine kinases (Fyn and Lck) (19-22 months).
       b.   Examine the effects of sarin on PLC-γ1 (23-26 months)
       c.   Investigate the effects of sarin on intracellular calcium stores (27-29 months).

Task 3.   To ascertain the role of the sympathetic autonomic nervous system in sarin-induced immunotoxicity (30-39 months).
       a.   Examine whether sarin activates the HPA axis (30-31 months).
       b.   Examine whether chemical or surgical sympathectomy abrogates the immunological effects of sarin (32-36 months).
       c.   Investigate whether β-adrenoceptor antagonists block the effects of sarin on T cell proliferation (37-38 months).

Task 4.   What roles do muscarinic and nicotinic acetylcholine receptors play in sarin-induced immunotoxicity? (39-48 months).
       a.   Determine the effects of sarin on changes in the acetylcholine esterase activity in various brain regions (39-40 months).
       b.   Establish whether sarin affects the density of nicotinic acetylcholine receptors in various brain regions (41-43 months).
       c.   Determine whether sarin affects the immune system in muscarinic or nicotinic acetylcholine receptor knockout mice (44-48 months).
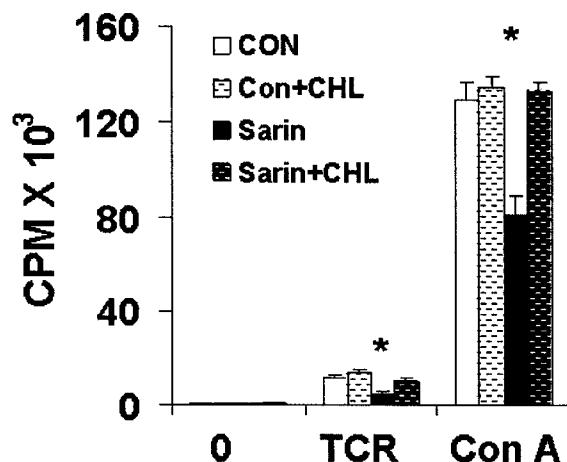
BODY:

1. **Neuroimmune effects of cholinergic compounds are mediated through the central nervous system (CNS):** We previously reported Kalra et al. (2002) that sarin-treated rats have significantly lower serum levels of corticosterone and decreased T-cell immunity, and these effects are mediated through the CNS. The mechanism of cholinergic immunotoxicity is largely unknown. To evaluate whether the immunological effects of cholinergic compounds is dependent on their ability to cross the blood-brain-barrier (BBB) and reach the CNS Task-1), rats were exposed chronically to cholinergic compounds via subcutaneous or intracerebroventricular routes. We observed that the compounds that cross the BBB inhibit the antibody response when given by either route, however, poorly permeable compounds, unless given in high doses, inhibited the antibody response only by intracerebroventricular administration. Intracerebroventricular administration of cholinergic agents also reduced serum corticosterone levels, which along with the antibody response was attenuated by pretreatment with the ganglionic blocker, chlorisondamine. Thus, cholinergic agents affect the neuroimmune communication and inhibit glucocorticoid production; the latter may be a biomarker for cholinergic toxicity. These results are presented in the paper by Langley et al. (2004; Raymond J. Langley, Roma Kalra, Neerad Mishra, Mohan L. Sopori. 2004. Central but not the peripheral action of cholinergic compounds suppresses the immune system. J. Neuroimmunol. 148: 140-145) (**Appendix-1**).

2. **Kinetics of the immunological changes associated with low-dose sarin exposure**: To determine the duration of immunological changes that are associated with low-dose exposure to sarin, F344 rats were exposed for 5 days (1 h/day) to low-dose (0.4 mg/m^3) sarin inhalation, as described previously (Kalra et al., 2002). Some animals received the ganglionic blocker, chlorisondamine, 7 days prior to sarin inhalation. Starting at 24 h, the animals were sacrificed at various times after the sarin treatment. The following results were obtained from these experiments:

    (a) <u>Effects of sarin on T cell proliferation are short-lived</u>.
   Splenic cells from control and sarin-treated animals were cultured with anti-TCR antibodies or the T cell mitogen, concanavalin-A (Con A) under conditions similar to those reported previously (Kalra et al., 2002; Langley et al., 2004). At 24 h after sarin exposure, sarin-treated cells had significantly lower proliferation than control (Fig. 1). At 1 wk and thereafter, the T cell proliferative response of sarin-treated animals was not significantly different from control animals(data not shown). Thus, the suppressive effects of sarin treatment on T cell proliferation were relatively short-lived and improved within a week of sarin exposure.


    (b) <u>Immunosuppressive effects of sarin on the antibody-forming cell (AFC) response remain for at least two wk.</u> Inhibition of the T-dependent antibody formation is one of the established criteria of immunosuppression (Luster et al., 1992). Fig. 2 shows that at 24 h, 1 wk, and 2 wk after sarin treatment, the AFC response to the T dependent antigen, sheep red blood cells (SRBC), is significantly lower than in control animals. At 4 wk, the difference is small, but statistically insignificant, and at 8 wk the response is essentially identical between the control and sarin-treated animals. Thus, for at least two wk, the AFC response of sarin-treated animals is lower than control animals.

AFC (1 Day)

AFC (1 wk)

AFC (2 wk)

AFC (4 wk)
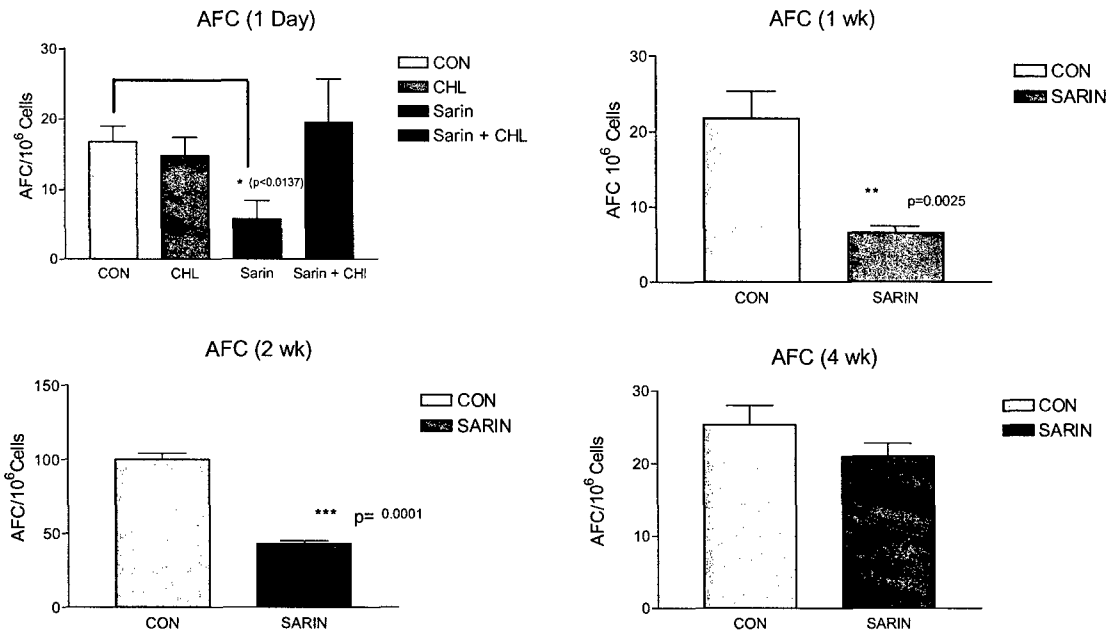
(c) <u>Sarin increases proinflammatory cytokines in the brain.</u> Excessive production of cytokines, such as IL-1β and TNF-α may promote inflammation at the site of cytokine production. Even mild increases in the concentration of these cytokines in the brain might produce symptoms such as headaches, myalgia, and nausea. Fig 3 shows that sarin treatment increases IL-1β and TNF-α in the brain. The increase is statistically significant at 1 wk after sarin treatment. Slight but statistically insignificant increases are also seen at 2 wk after the treatment.

IL-1β in the Brain 1 week

IL-1β in the Brain 2 week

TNF-α in the Brain 1 week

TNF-α in the Brain 2 week
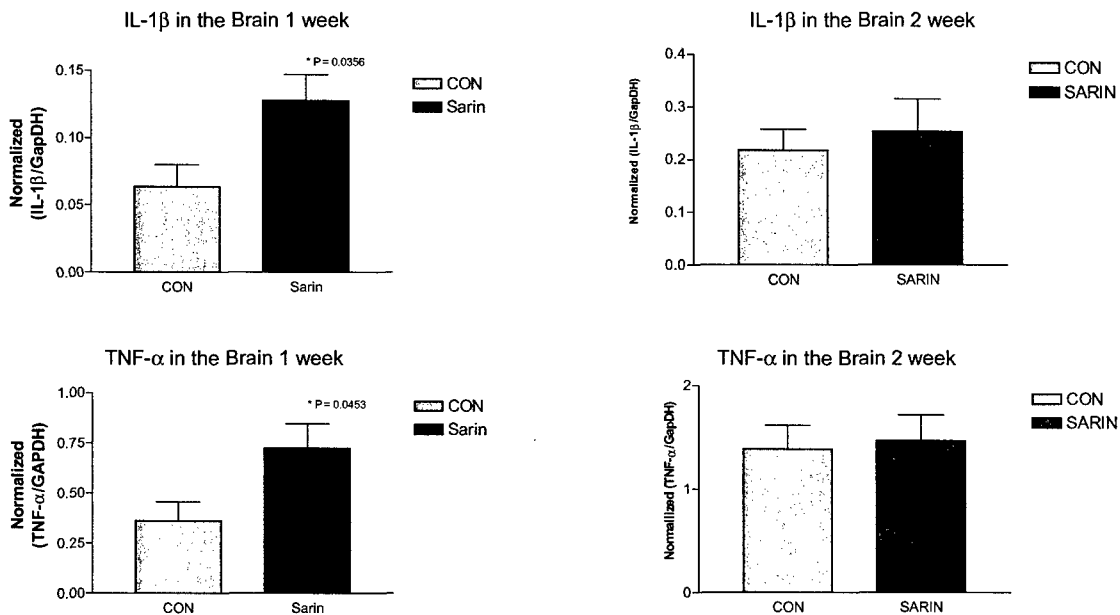
(d) <u>Depressed glucocorticoid production is a long term effect of sarin exposure.</u> Serum corticosterone levels are dramatically decreased in sarin-treated rats (Kalra et al., 2002), and this appears to be a generalized property of cholinergic neurotoxicity (Langley et al., 2004). However, the previous studies were carried out immediately after cholinergic treatment, and it was not clear whether the corticosterone production is restored after the cholinergic agent is cleared from the body. Fig. 4 shows that decreased serum corticosterone levels in sarin-treated rats are seen at least until 8 wk after sarin treatment. Thus, decreased glucocorticoid level may be a biomarker for cholinergic neurotoxicity.

Corticosterone ng/mL

750 —

500 —

* (p < 0.0326)

* (p<0.0407)

**(p< 0.0098)

250 —

0 —

CON   SAR 1 day SAR 2 Wk SAR 8 Wk
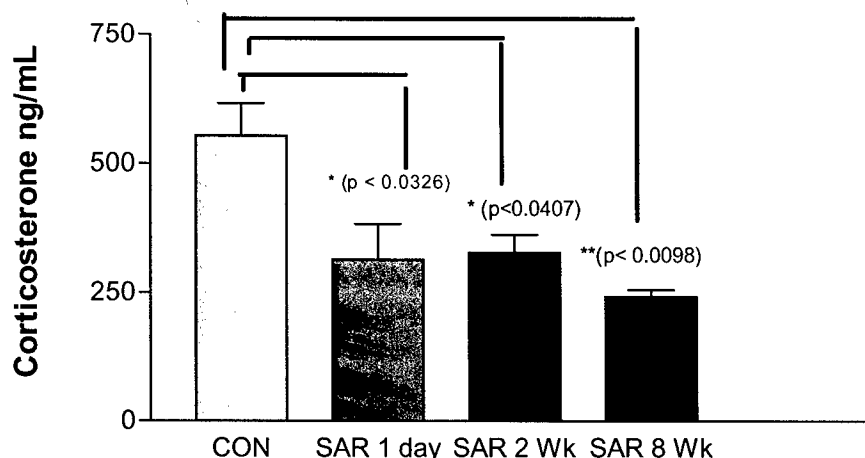
(e) <u>Sarin may affect several intracellular signaling pathways.</u> Because sarin treatment inhibits the ability of T cells to raise the intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in response to TCR ligation, and increases the proinflammatory cytokine levels in the brain, we examined the signaling pathways in T cells that control these responses. Very preliminary experiments (data not shown) indicate that sarin exposure impairs the protein tyrosine kinase (PTK) pathway that controls the antigen-mediated Ca^{2+} response, as well as the MAP-kinase pathway that controls the activation of the transcription factor NFκB and expression of proinflammatory cytokines.

LEGENDS TO FIGURES

Figure 1: <u>Effects of sarin T cell proliferation are short-lived.</u> Animals were exposed to sarin inhalation 1 h/d for 5 days. Twenty four after sarin exposure, spleen cells were isolated from control and sarin-treated rats and cultured for 3 days in microtiter wells in the presence of indicated concentrations of anti-αβ-TCR or 1 µg/ml Con A (Kalra et al., 2002). Eighteen hours before harvesting, culture wells were pulsed with 0.5 µCi of [^{3}H]-thymidine, and harvested cells counted in a β-counter. Some animals were pretreated with chlorisondamine (CHL) prior to sarin exposures, as described previously (Kalra et al., 2002). Results represent mean and SD values from 5 animals per group.

Figure 2: <u>Sarin inhibits the AFC response for at least 2 wk after treatment.</u> Animals were immunized with SRBC at indicated times after sarin exposure. Anti-SRBC AFC response of the spleen cells was determined 4 days after the immunization. Some animals were pretreated with chlorisondamine (CHL) prior to sarin exposure.

Figure 3: <u>Sarin increases TNF-α and IL-1β levels in the brain.</u> RNAs were isolated from control and sarin-treated animals (5 animals/group at various times after sarin treatment). RNA from each animal brain was analyzed for IL-1β and TNF-α expression by real-time PCR.

Figure 4: <u>Sarin produces long-term effects on serum corticosterone levels.</u> Serum was isolated from animals at indicated times after sarin treatment. Serum corticosterone levels were quantitated with a radioimmunoassay kit (Langley et al., 2004).

KEY RESEARCH ACCOMPLISHMENTS
- Exposure to subclinical doses of sarin suppresses the immune system and glucocorticoid production, and the effects are at least partially ameliorated by pretreatment with ganglionic blockers.
- Cholinergic agents that cross the blood-brain-barrier cause immunotoxicity similar to sarin.
- Most effects of sarin are temporary (i.e., lasting for 1-2 wk), however, the suppressive effects on glucocorticoid production may be relatively long-term (i.e., last at least for 8 wk post sarin exposure).

- Preliminary results indicate that sarin may affect antigen receptor-mediated and MAP-kinase signaling cascade in T cells. If confirmed, sarin-induced effects on these pathways may explain its effects on the Ca^{2+} response and production of proinflammatory cytokines.
- Ganglionic blockers may have therapeutic value in the treatment of cholinergic immunotoxicity, and decreased serum glucocorticoid level is a potential biomarker for exposure to sarin and other BBB-crossing cholinergic agents.

REPORTABLE OUTCOMES

1. Langley, R.J., R. Kalra, N.M. Mishra, M.L. Sopori. 2004. Central but not the peripheral action of cholinergic compounds suppresses the immune system. J. Neuroimmunol. 148: 140-145.

2. Pena-Philippides, J.C., N.M. Mishra, Langley, R.J., S. Razani-Boroujerdi, R. Kalra, S.P. Singh, and M.L. Sopori. Kinetics of sarin immunotoxicity: I. T cell function is restored within 2-4 weeks post exposure, but changes in the serum glucocorticoid level are long-term. (Manuscript in preparation).

CONCLUSIONS

Subclinical exposure to cholinergic agents, such as sarin, pesticides, and other organophosphates suppress the immune system, and this immunotoxicity is dependent on their ability to cross the BBB. The effects are mediated through the autonomic nervous system and are at least partially overcome by ganglionic blockers. Cholinergic neurotoxicity also suppresses glucocorticoid production; this effect is relatively long-term and may be a biomarker for cholinergic toxicity. The Increased brain levels of proinflammatory cytokines might cause some early symptoms of the Gulf War syndrome, and ganglionic blockers may have some therapeutic values.

REFERENCES

Kalra, R., Shashi P. Singh, Seddigheh Razani-Boroujerdi, Raymond J. Langley, Walter B. Blackwell, Rogene F. Henderson, and Mohan L. Sopori. 2002. Subclinical doses of the nerve gas sarin impair T cell responses through the autonomic nervous system. Toxicol. Appl. Pharmacol. 184: 82-87.

Langley, Raymond J., Roma Kalra, Neerad Mishra, Mohan L. Sopori. 2004. Central but not the peripheral action of cholinergic compounds suppresses the immune system. J. Neuroimmunol. 148: 140-145.

Luster, M.I., C. Porteir, D.G. Pait, K.L. White, C. Gennings, A.E. Munson, G.J. Rosenthal. 1992. Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. Fundam. Appl. Toxicol. 18: 200-210.

APPENDICES

1. Langley, Raymond J., Roma Kalra, Neerad Mishra, Mohan L. Sopori. 2004. Central but not the peripheral action of cholinergic compounds suppresses the immune system. J. Neuroimmunol. 148: 140-145.

ELSEVIER

Central but not the peripheral action of cholinergic compounds suppresses the immune system

Raymond J. Langley, Roma Kalra, Neerad C. Mishra, Mohan L. Sopori*

Immunology Program, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr., S.E., Albuquerque, NM 87108, USA

Abstract

Cholinergic compounds modulate the immune system; however, the mechanism of cholinergic immunotoxicity is largely unknown. Lewis rats were exposed chronically to cholinergic compounds via subcutaneous or intracerebroventricular routes. Compounds that crossed the blood–brain barrier (BBB) inhibited the antibody response when given by either route, however, poorly permeable compounds, unless given in high doses, inhibited the antibody response only by intracerebroventricular administration. Intracerebroventricular administration of cholinergic agents also reduced serum corticosterone levels, which along with the antibody response was attenuated by pretreatment with the ganglionic blocker chlorisondamine. Thus, cholinergic agents affect the neuroimmune communication and inhibit glucocorticoid production; the latter may be a biomarker for cholinergic toxicity.
© 2004 Elsevier B.V. All rights reserved.

1. Introduction

Cholinergic agents including organophosphates and myasthenia gravis therapeutics (e.g., pyridostigmine bromide (PB), physostigmine, edrophonium) elevate the synaptic levels of acetylcholine (Grob and Harvey, 1958). PB was also used by the U.S. Army as a prophylactic against potential nerve gas attack during the first Gulf War (Roberts et al., 1994). Organophosphates such as pesticides, including malathion, parathion, and dichlorvos (Street and Sharma, 1975; Desi et al., 1978; Casale et al., 1983) and the nerve gas agent, sarin (Kalra et al., 2002) impair cellular and humoral immune responses in animal models. Mounting evidence suggests an intimate relationship between the neuroendocrine and immune system (reviewed in Blalock, 1994) and neuroactive substances might affect the immune system through the central nervous system (CNS). We have demonstrated that some of the effects of nicotine and sarin on the immune system are mediated through the CNS (Sopori et al., 1998; Sopori, 2002; Kalra et al., 2002). Moreover, the ganglionic blocker chlorisondamine (CHL) attenuates the inhibition of the antibody-forming cell (AFC) response by chronic low-dose sarin (Kalra et al., 2002). Thus, cholinergic agents may affect the immune system through the autonomic nervous system. To ascertain whether the neuroimmune effects of a cholinergic agent require its entry into the CNS, in this communication we used cholinergic compounds that do or do not cross the blood–brain barrier (BBB), and demonstrate that cholinergic agents may have some common biological effects, such as suppression of the immune system and glucocorticoid production, and the immunomodulatory effects of cholinergic agents are predicated on their entry into the CNS.

2. Materials and methods

2.1. Animals

Six- to eight-week-old, pathogen-free, male Lewis (LEW) rats were purchased from Charles River (Raleigh, NC, USA). The animals were housed in class-100 air quality rooms in shoebox cages with hardwood chip bedding. Food and water were provided ad lib, and animals were periodically monitored for common rat infections.

---

* Corresponding author. Tel.: +1-505-348-9440; fax: +1-505-348-4986.

E-mail address: msopori@lrri.org (M.L. Sopori).

2.2. Reagents

Unless otherwise mentioned, all the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. CHL treatment

The ganglionic blocker CHL (Tocris, Ballwin, MO, USA) was injected subcutaneously into rats (10 mg/kg body wt) 7 days prior to treatment with cholinergic agents. This concentration of CHL blocks the behavioral responses to neuroactive substances for several months (Reuben et al., 1998). It was determined previously that under these conditions CHL per se had virtually no effect on the AFC or T-cell proliferative responses in control animals (Kalra et al., 2002).

2.4. Treatment with cholinergic agents

For implantation of Alzet miniosmotic pumps (Alzet, Palo Alto, CA, USA), rats were anesthetized with an intramuscular injection of a mixture of ketamine and xylazine (87 mg and 13 mg/kg body wt, respectively). Twenty-eight-day miniosmotic pumps (Model 2ML4) were implanted subcutaneously as described previously (Geng et al., 1995). The pumps provided saline (control) and indicated concentrations of physostigmine, PB, or edrophonium. For intracerebroventricular (i.c.v.) administration, rats were anesthetized with ketamine and xylazine as described above, and stereotaxically implanted with 5-mm-long 28-gauge steel cannulae connected to a 2ML4 miniosmotic pump (Alzet Brain Infusion Kit) according to the manufacturer's instructions (Sopori et al., 1998). The coordinates were 1 mm posterior to the bregma and 1.5 mm to the midline (Pellegrino et al., 1979). Control miniosmotic pumps contained artificial cerebrospinal fluid (α-CSF) composed of 145 mM NaCl, 3.3 mM KCl, 1.3 mM $CaCl_2$, and 1.0 mM $MgCl_2$ dissolved in pyrogen-free water and filtered through a 0.2-μm membrane filter (Klir et al., 1995). The pumps were filled with α-CSF, PB (0.5 mg/kg/day), physostigmine (0.02 mg/kg/day), or edrophonium (1.5 mg/kg/day) dissolved in α-CSF. Animals were sacrificed 3 weeks after the implantation.

2.5. Immunizations

For determining the AFC response, rats were injected intravenously with (5×10^8) sheep red blood cells (SRBC) 4 days prior to sacrifice as previously described (Sopori et al., 1989).

2.6. Preparation of spleen cells

Spleen cell suspensions were prepared as described (Sopori et al., 1985). Briefly, spleens were pressed through a stainless-steel mesh, and red blood cells were lysed by treatment with a cold NH_4Cl solution. After the centrifu-gation, cells were washed twice with cold PBS, and viable cells were counted in 0.1% eosin on a hemocytometer.

2.7. AFC assay

The primary direct AFC response was determined essentially as described by Cunningham and Szenberg (1968). Briefly, spleen cells (2×10^5) were mixed with 2% SRBC and 25 μl of guinea pig complement (Cederlane, Hornby, ON, Canada) pre-absorbed on SRBC in a final volume of 250 μl of complete medium (RPMI 1640 containing 10% fetal calf serum, 2 mM glutamine, 50 μM 2-ME, and 10 μg/ml gentamicin). Aliquots were distributed in duplicates on Cunningham slides and incubated for 45 min at 37 °C. The AFC plaques were counted and normalized to AFC/10^6 spleen cells and expressed as percent of control.

2.8. T-cell proliferation assay

The proliferative response of spleen cells to the T-cell mitogen, Concanavalin A (Con A), was measured as previously described (Sopori et al., 1987). Briefly, 2×10^5 spleen cells were cultured in 0.2 ml of the complete medium in the presence of various concentrations of Con A in microtiter plate wells. The cultures were incubated at 37 °C in the presence of 5% CO_2, and cells were harvested after 3 days by a Skatron cell harvester (Skatron, Sterling, VA, USA). T cell proliferation was assayed by adding 0.5 μCi of [^{3}H] thymidine (ICN, Irvine, CA, USA) to the culture wells 18 h before harvesting the cells.

2.9. Assay for serum corticosterone levels

Serum corticosterone levels were determined by the corticosterone RIA-kit (ICN Biochemicals, Orangeburg, NY, USA.) according to the manufacturer's instructions. As stated by the manufacturer, under these assay conditions, normal rat serum corticosterone levels vary from 50 to 400 ng/ml.

2.10. Statistical analysis

Data was analyzed for statistical significance by the Prism Software 3.0 (Graphpad, San Diego, CA, USA) using a Student's t-test. Values were considered significant at $p \leq 0.05$.

3. Results

3.1. Chronic subcutaneous administration of physostigmine, but not PB or edrophonium inhibits the AFC response

Physostigmine is a tertiary amine and crosses the BBB, while PB and edrophonium are quaternary compounds that do not ordinarily cross the BBB in appreciable quantities. However, under certain conditions such as stress and high

concentrations, they might cross the BBB (Friedman et al., 1996; Abdel-Rahman et al., 2002). To ascertain the effects of these agents on the immune system when administered peripherally at low doses, rats were treated daily via subcutaneously implanted miniosmotic pumps with saline (control), physostigmine (0.2 mg/kg/day), PB (0.5 mg/kg/day), or edrophonium (1.5 mg/kg/day) for 3 weeks. Four days prior to sacrifice, animals were injected intravenously with SRBC and the splenic AFC response was determined. Fig. 1 shows that under these conditions only physostigmine significantly suppressed the anti-SRBC AFC response, but PB and edrophonium were essentially ineffective. Raising the concentration of PB and edrophonium by approximately fivefold (i.e., 2.4 and 7.2 mg/kg/day, respectively) did not lead to a significant inhibition of the AFC (Fig. 2) or the splenic Con A-induced proliferative response (data not shown) by PB; however, edrophonium caused a small but significant decrease in the AFC response. The decrease in the AFC response by edrophonium was surprising; however, it is possible that at high concentrations small quantities of edrophonium might gain entry into the CNS, or some of the effects of edrophonium might be unrelated to its inhibition of the brain cholinesterase activity (Matteo et al., 1990). Thus, unless administered at high concentrations, peripheral administration of BBB impermeable cholinergic agents does not affect the immune system.

3.2. I.C.V. administration of PB and edrophonium inhibits AFC response

Unlike PB and edrophonium, physostigmine crosses the BBB under normal conditions. To determine whether the effects of cholinergic compounds on the immune
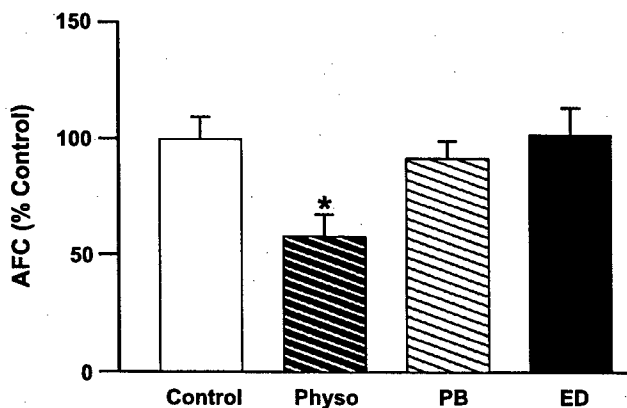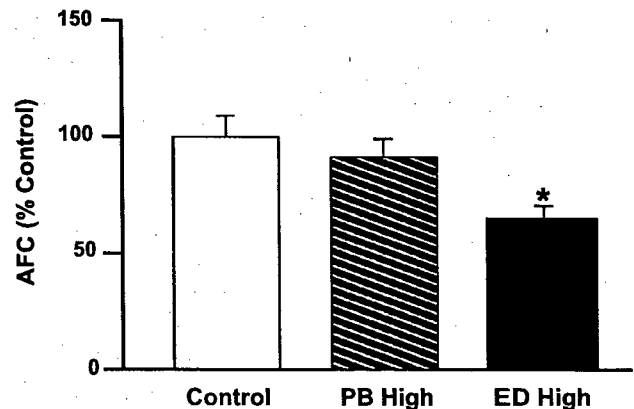
Fig. 2. Subcutaneous (s.c.) administration of high concentration of edrophonium (ED) but not of PB inhibited the AFC response. Animals (5/group) were treated subcutaneously with saline (control), PB (2.4 mg/kg/day), or edrophonium (7.2 mg/kg/day) for 3 weeks. The AFC response was determined as in Fig. 1. Differences between control and edrophonium were significant ($p = 0.011$).

system required their entrance into the CNS, we determined whether chronic treatment with low concentrations of these compounds directly into the brain (i.e., i.c.v. administration) affected the immune system. Rats were treated for 3 weeks i.c.v. with approximately 10% of the concentration of physostigmine that caused significant inhibition of the AFC response by subcutaneous route (i.e., 0.02 mg physostigmine/kg/day). For i.c.v. administration, the concentrations of PB and edrophonium used were 0.5 and 1.5 mg/kg/day, respectively. Fig. 3 shows that when given in concentrations that did not affect the AFC response through subcutaneous administration, PB and edrophonium significantly inhibited the anti-SRBC AFC response if administered via i.c.v. Moreover, even at tenfold lower concentrations, i.c.v. administration of phy-
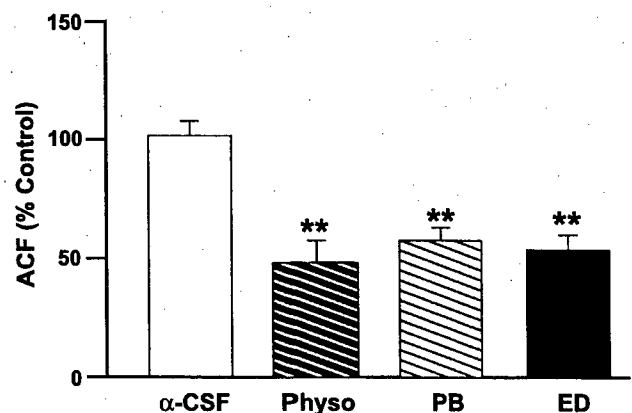
Fig. 1. Subcutaneous administration of physostigmine (physo), but not edrophonium (ED) or PB, inhibited the AFC response. Animals (N=5/group) were treated with saline (control), physostigmine (0.2 mg/kg/day), PB (0.5 mg/kg/day) or edrophonium (1.5 mg/kg/day) via subcutaneously implanted miniosmotic pumps for 3 weeks. Four days prior to sacrifice, animals were injected intravenously with SRBC and anti-SRBC AFC response determined (see Materials and methods). The data is represented as percent of control. Statistical significance: differences between control and PB or edrophonium were statistically insignificant, whereas the difference for physostigmine was significant ($p = 0.012$).

Fig. 3. I.c.v. administration of low doses of physostigmine (Physo), PB, and edrophonium (ED) suppress the anti-SRBC AFC response. Animals (N=5/group) were treated with α-CSF (control), physostigmine (0.02 mg/kg/day), PB (0.5 mg/kg/day) or edrophonium (1.5 mg/kg/day) for 3 weeks via i.c.v. administration. Values for α-CSF (i.c.v.) were comparable to s.c. saline control. Animals were immunized with SRBC and AFC response determined as in Fig. 1. Control values were statistically different from: physostigmine ($p = 0.003$), PB ($p = 0.001$), and edrophonium (0.001).
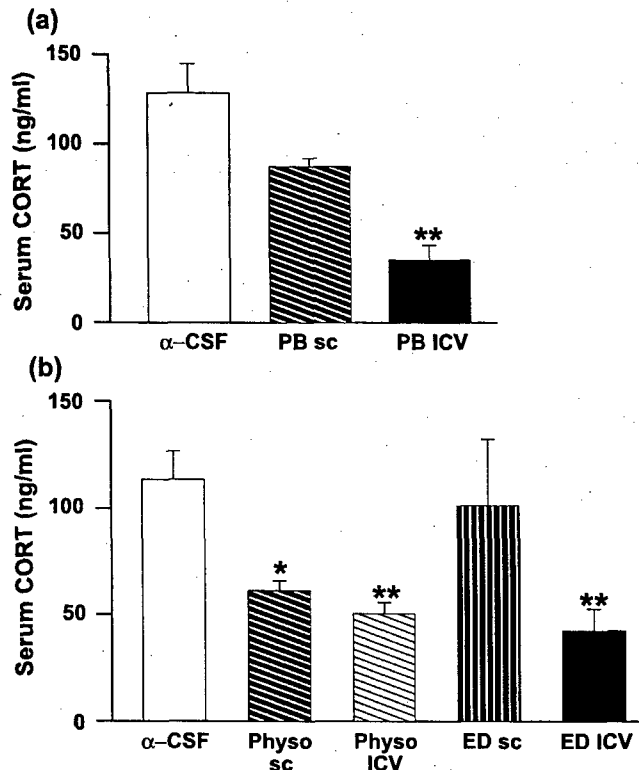
Fig. 4. I.c.v. administration of physostigmine (Physo), PB, and edrophonium (ED) suppress serum corticosterone (CORT) levels. Animals (5/group) were treated for 3 weeks with saline or α-CSF as controls for subcutaneous (s.c.) or i.c.v. cholinergic treatments, respectively—all of the responses were similar (data not shown) and control values were given as α-CSF. (a) PB, 2.4 mg/kg/day s.c. or 0.5 mg/kg/day i.c.v.; (b) physostigmine (s.c., 0.2 mg/kg/day; i.c.v., 0.02 mg/kg/day), edrophonium (s.c., 7.2 mg/kg/day; i.c.v., 1.5 mg/kg/day). Statistical differences: PB (s.c., insignificant; i.c.v., $p = 0.002$), edrophonium (s.c., insignificant; i.c.v., $p = 0.002$), physostigmine (s.c., $p = 0.013$; i.c.v., $p = 0.005$).

sostigmine strongly inhibited the AFC response. Therefore, it is likely that the immunosuppressive effects of cholinergic agents require their entry into the CNS.

3.3. Immunosuppressive effects of cholinergic agents are not controlled through increased production of glucocorticoids

The hypothalamus–pituitary–adrenal (HPA) modulates the immune response through elevation of glucocorticoids (Turnbull and Rivier, 1999). However, sarin (a potent cholinergic agent) does not stimulate but actually strongly suppresses the serum corticosterone levels in rats (Kalra et al., 2002). To determine whether inhibition of corticosterone production is a general property of cholinergic agents, rats were treated subcutaneously or i.c.v. with PB, physostigmine, or edrophonium. Data presented in Fig. 4a shows that i.c.v. but not subcutaneous administration of low doses of PB decreased the serum corticosterone levels. Similarly, only i.c.v. administration of edrophonium decreased the serum corticosterone levels (Fig. 4b). However, physostigmine, which crosses the BBB, was effective in decreasing

the serum corticosterone level by either i.c.v. or subcutaneous administration (Fig. 4b). Thus, serum corticosterone levels are primarily modulated by the central activity of cholinergic compounds.

3.4. CHL attenuates PB-induced immunosuppression and decrease in serum corticosterone levels

To examine whether PB affected the immune system through the autonomic nervous system, animals were pretreated with CHL 7 days prior to a 3-week i.c.v. exposure to PB. Fig. 5a shows that the anti-SRBC response of PB-treated animals was significantly lower than α-CSF-treated animals, and that this decrease in the AFC response was partially moderated by treatment with CHL ($p = 0.058$). Similarly, the PB-induced drop in the serum corticosterone levels was lower in PB-treated animals that were pretreated with CHL (Fig. 5b). Combined with previously published
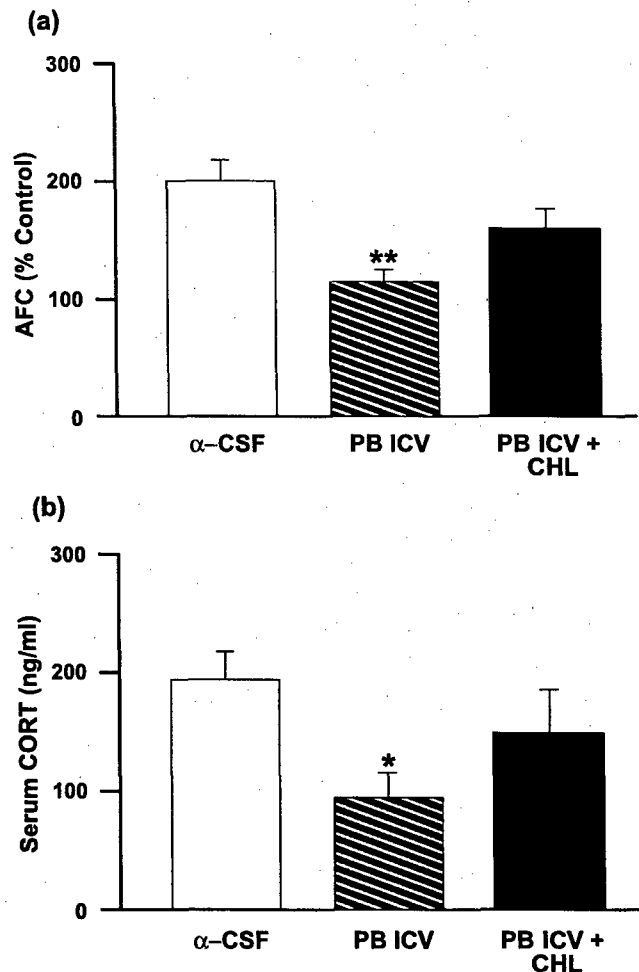

Fig. 5. CHL attenuates the inhibitory effects of i.c.v. PB administration on the AFC response (a) and serum corticosterone (CORT) levels. Animals (5/group) were pretreated with CHL 7 days prior to 3-week treatment with α-CSF or PB (i.c.v., 0.5 mg/kg/day) as described in Materials and methods. (a) Effects on the AFC response ($p = 0.007$ between control and PB; $p = 0.058$ between PB and PB + CHL); (b) effects on CORT levels ($p = 0.027$ between control and PB).

results on sarin (Kalra et al., 2002), these data suggest that the autonomic nervous system may play an important role in mediating the immunotoxicity of cholinergic agents.

4. Discussion

Organophosphates such as pesticides and the nerve gas agent sarin irreversibly inactivate acetylcholine esterase and increase the synaptic level of acetylcholine (Grob and Harvey, 1958). Reversible cholinesterase inhibitors (such as PB, physostigmine, and edrophonium) compete with irreversible cholinesterase inhibitors and are used as therapeutics to increase the synaptic levels of acetylcholine in myasthenia gravis patients; PB also was used prophylactically for potential nerve gas exposure during the first Gulf War (Roberts et al., 1994). Several cholinergic agents have been shown to suppress the immune system; however, the mechanism of immunosuppression is not clear. Results presented herein show that, if given i.c.v., reversible cholinesterase inhibitors such as physostigmine, edrophonium, and PB suppress the immune system; thus, immunosuppression may be a common outcome of the central cholinergic toxicity.

Organophosphates are neuroactive substances, and neuroactive compounds including opiates (Nelson and Lysle, 2001), nicotine (Sopori et al., 1998; Sopori, 2002), and sarin (Kalra et al., 2002) affect the immune system through the CNS. The neuroendocrine and immune systems are intimately related during development, maturation, and the aging process (Heijnen and Kavelaars, 1999). The two systems communicate bidirectionally through hormones, cytokines, and neurotransmitters via several pathways (Blalock, 1994). The HPA axis communicates with the periphery through the release of anterior and posterior pituitary hormones that, among other effects, lead to the production of glucocorticoids from the adrenals that inhibit the immune responses (Turnbull and Rivier, 1999). The other established pathway for neuroimmune communication is the autonomic nervous system, which connects the CNS directly to visceral target tissues via the sympathetic and parasympathetic nerves (Felten and Felten, 1994; Borovikova et al., 2000). The sympathetic nerve fibers are in direct contact with T-cells in the white pulp of the spleen (Felten and Felten, 1994). To ascertain whether the immunomodulation by cholinergic agents required the CNS entry by these agents, we examined the immunosuppressive effects of the BBB permeable (physostigmine) and BBB impermeable (PB and edrophonium) cholinergic agents. Clearly, at low concentrations, all the compounds were active when given i.c.v. Subcutaneous administration of low doses of physostigmine resulted in decreased AFC response, while PB and edrophonium were essentially inactive under these conditions. Moderately high concentrations of edrophonium, but not PB, caused a small but significant reduction in the AFC response. These effects of

edrophonium may result from some of its unusual properties. For example, the duration of acetylcholine esterase inhibition by edrophonium is not tightly linked to its plasma concentrations, and it has been hypothesized that its biological effects might not totally result from its inhibition of cholinesterase activities (Blaber and Bowman, 1959; Matteo et al., 1990). Moreover, indirect evidence suggests that edrophonium may act at a site different from neostigmine and PB (Cronnelly et al., 1982). Thus, it is not clear whether the suppression of the AFC response by high doses of edrophonium results from changes in the BBB through overloading and stress (Friedman et al., 1996; Abdel-Rahman et al., 2002) or through non-central effects of high-dose toxicity. However, it is clear that, in general, the immunomodulatory effects of a cholinergic agent are dependent on its entry into the CNS.

Experiments to ascertain whether the inhibition of the AFC response by i.c.v. administration of PB, physostigmine, and edrophonium is mediated by glucocorticoids through the activation of the HPA axis indicated unequivocally that these compounds do not stimulate glucocorticoid production; in fact, these cholinergic agents are potent inhibitors of corticosterone production. These results, together with those observed with sarin (Kalra et al., 2002), clearly suggest that cholinergic agents do not suppress the immune system through increased glucocorticoid production; on the other hand decreased glucocorticoid production may be a biomarker for cholinergic toxicity.

To evaluate whether cholinergic agents affected the immune system through the autonomic nervous system, animals were pretreated with the ganglionic blocker CHL prior to exposure to PB. Results suggested that CHL treatment attenuated the suppression of the AFC response as well as that of serum corticosterone levels. However, the reversal was not complete, and might reflect incomplete inhibition of the autonomic nervous system by the dose of CHL used in these studies. Because subcutaneous administration of PB tended to decrease, although not significantly, the serum corticosterone levels without affecting the AFC response, it suggests that either the adrenal responses are more sensitive than the immune responses to cholinergic toxicity or that corticosterone production is controlled both by the central as well as peripheral activities of cholinergic agents. While additional studies are needed to sort out this ambiguity, it is clear that the autonomic nervous system plays an important role in the immunomodulatory effects of cholinergic agents.

Acknowledgements

be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation. In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985). The Lovelace Respiratory Research Institute is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

References

Abdel-Rahman, A., Shetty, A.K., Abou-Donia, M.B., 2002. Disruption of the blood–brain barrier and neuronal cell death in cingulate cortex, dentate gyrus, thalamus, and hypothalamus in a rat model of Gulf-War syndrome. Neurobiol. Dis. 10, 306–326.

Blaber, L.C., Bowman, W.C., 1959. A comparison between the effects of edrophonium and choline in skeletal muscles of the cat. Br. J. Pharmacol. 14, 456–466.

Blalock, J.E., 1994. The syntax of immune-neuroendocrine communication. Immunol. Today 15, 504–511.

Borovikova, L.V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G.I., Watkins, L.R., Wang, H., Abumrad, N., Eaton, J.W., Tracey, K.J., 2000. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405, 458–562.

Casale, G.P., Cohen, S.D., DiCapua, R.A., 1983. The effects of organophosphate-induced cholinergic stimulation on the antibody response to sheep erythrocytes in inbred mice. Toxicol. Appl. Pharmacol. 68, 198–205.

Cronnelly, R., Morris, R.B., Miller, R.D., 1982. Edrophonium: duration of action and atropine requirement in humans during halothane anesthesia. Anesthesiology 57, 261–266.

Cunningham, A.J., Szenberg, A., 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology 14, 599–600.

Desi, I., Varga, L., Farkas, I., 1978. Studies on the immunosuppressive effect of organochlorine and organophosphoric pesticides in subacute experiments. J. Hyg. Epidemiol. Microbiol. Immunol. 22, 115–122.

Felten, S.Y., Felten, D.L., 1994. Neural-immune interactions. Prog. Brain Res. 100, 157–162.

Friedman, A., Kaufer, D., Shemer, J., Hendler, I., Soreq, H., Tur-Kaspa, I., 1996. Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. Nat. Med. 2, 1382–1385.

Geng, Y., Savage, S.M., Johnson, L.J., Seagrave, J., Sopori, M.L., 1995. Effects of nicotine on the immune response: I. Chronic exposure to nicotine impairs antigen receptor-mediated signal transduction in lymphocytes. Toxicol. Appl. Pharmacol. 135, 268–278.

Grob, D., Harvey, J.C., 1958. Effects in man of the anticholinesterase compound sarin (isopropyl methylphosphonofluoridate). J. Clin. Invest. 37, 350–368.

Heijnen, C.J., Kavelaars, A., 1999. The importance of being receptive. J. Neuroimmunol. 100, 197–202.

Kalra, R., Singh, S.P., Razani-Boroujerdi, S., Langley, R.J., Blackwell, W.B., Henderson, R.F., Sopori, M.L., 2002. Subclinical doses of the nerve gas sarin impair T cell responses through the autonomic nervous system. Toxicol. Appl. Pharmacol. 184, 82–87.

Klir, J.J., McClellan, J.L., Kozak, W., Szelenyi, Z., Wong, G.H., Kluger, M.J., 1995. Systemic but not central administration of tumor necrosis factor-alpha attenuates LPS-induced fever in rats. Am. J. Physiol. 268, R480–R486.

Matteo, R.S., Young, W.L., Ornstein, E., Schwartz, A.E., Silverberg, P.A., Diaz, J., 1990. Pharmacokinetics and pharmacodynamics of edrophonium in elderly surgical patients. Anesth. Analg. 71, 334–339.

Nelson, C.J., Lysle, D.T., 2001. Involvement of substance P and central opioid receptors in morphine modulation of the CHS response. J. Neuroimmunol. 115, 101–110.

Pellegrino, L., Pellegrino, A.S., Cushman, A.J., 1979. A Stereotaxic Atlas of the Rat Brain, 2nd ed. Plenum, New York.

Reuben, M., Louis, M., Clarke, P.B., 1998. Persistent nicotinic blockade by chlorisondamine of noradrenergic neurons in rat brain and cultured PC12 cells. Br. J. Pharmacol. 125, 1218–1227.

Roberts, D.E., Sawka, M.N., Young, A.J., Freund, B.J., 1994. Pyridostigmine bromide does not alter thermoregulation during exercise in cold air. Can. J. Physiol. Pharm. 72, 788–793.

Sopori, M., 2002. Effects of cigarette smoke on the immune system. Nat. Rev., Immunol. 2, 372–377.

Sopori, M.L., Perrone, R.S., Cherian, S., Cross, R.J., Kaplan, A.M., 1985. Immunoregulation in the rat: characteristics of a suppressor T cell that inhibits antigen-dependent cell proliferation. J. Immunol. 135, 80–86.

Sopori, M.L., Hurt, Y.L., Cherian, S., Kaplan, A.M., Diamantstein, T., 1987. Differential requirement for accessory cells in polyclonal T-cell activation. Cell. Immunol. 105, 174–186.

Sopori, M.L., Cherian, S., Chilukuri, R., Shopp, G.M., 1989. Cigarette smoke causes inhibition of the immune response to intratracheally administered antigens. Toxicol. Appl. Pharmacol. 97, 489–499.

Sopori, M.L., Kozak, W., Savage, S.M., Geng, Y., Soszynski, D., Kluger, M.J., Perryman, E.K., Snow, G.E., 1998. Effect of nicotine on the immune system: possible regulation of immune responses by central and peripheral mechanisms. Psychoneuroendocrinology 23, 189–204.

Street, J.C., Sharma, R.P., 1975. Alteration of induced cellular and humoral immune responses by pesticides and chemicals of environmental concern: quantitative studies of immunosuppression by DDT, aroclor 1254, carbaryl, carbofuran, and methylparathion. Toxicol. Appl. Pharmacol. 32, 587–602.

Turnbull, A.V., Rivier, C.L., 1999. Regulation of the hypothalamic–pituitary–adrenal axis by cytokines: actions and mechanisms of action. Physiol. Rev. 79, 1–71.

Central but not the peripheral action of cholinergic compounds suppresses the immune system

Raymond J. Langley, Roma Kalra, Neerad C. Mishra, Mohan L. Sopori*

Immunology Program, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr., S.E., Albuquerque, NM 87108, USA

Abstract

Cholinergic compounds modulate the immune system; however, the mechanism of cholinergic immunotoxicity is largely unknown. Lewis rats were exposed chronically to cholinergic compounds via subcutaneous or intracerebroventricular routes. Compounds that crossed the blood–brain barrier (BBB) inhibited the antibody response when given by either route, however, poorly permeable compounds, unless given in high doses, inhibited the antibody response only by intracerebroventricular administration. Intracerebroventricular administration of cholinergic agents also reduced serum corticosterone levels, which along with the antibody response was attenuated by pretreatment with the ganglionic blocker chlorisondamine. Thus, cholinergic agents affect the neuroimmune communication and inhibit glucocorticoid production; the latter may be a biomarker for cholinergic toxicity.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Immunotoxicity; Cholinergic agents; Immunosuppression; Glucocorticoids

1. Introduction

Cholinergic agents including organophosphates and myasthenia gravis therapeutics (e.g., pyridostigmine bromide (PB), physostigmine, edrophonium) elevate the synaptic levels of acetylcholine (Grob and Harvey, 1958). PB was also used by the U.S. Army as a prophylactic against potential nerve gas attack during the first Gulf War (Roberts et al., 1994). Organophosphates such as pesticides, including malathion, parathion, and dichlorvos (Street and Sharma, 1975; Desi et al., 1978; Casale et al., 1983) and the nerve gas agent, sarin (Kalra et al., 2002) impair cellular and humoral immune responses in animal models. Mounting evidence suggests an intimate relationship between the neuroendocrine and immune system (reviewed in Blalock, 1994) and neuroactive substances might affect the immune system through the central nervous system (CNS). We have demonstrated that some of the effects of nicotine and sarin on the immune system are mediated through the CNS (Sopori et al., 1998; Sopori, 2002; Kalra et al., 2002). Moreover, the

ganglionic blocker chlorisondamine (CHL) attenuates the inhibition of the antibody-forming cell (AFC) response by chronic low-dose sarin (Kalra et al., 2002). Thus, cholinergic agents may affect the immune system through the autonomic nervous system. To ascertain whether the neuroimmune effects of a cholinergic agent require its entry into the CNS, in this communication we used cholinergic compounds that do or do not cross the blood–brain barrier (BBB), and demonstrate that cholinergic agents may have some common biological effects, such as suppression of the immune system and glucocorticoid production, and the immunomodulatory effects of cholinergic agents are predicated on their entry into the CNS.

2. Materials and methods

2.1. Animals

Six- to eight-week-old, pathogen-free, male Lewis (LEW) rats were purchased from Charles River (Raleigh, NC, USA). The animals were housed in class-100 air quality rooms in shoebox cages with hardwood chip bedding. Food and water were provided ad lib, and animals were periodically monitored for common rat infections.

* Corresponding author. Tel.: +1-505-348-9440; fax: +1-505-348-4986.
 E-mail address: msopori@lrri.org (M.L. Sopori).

2.2. Reagents

Unless otherwise mentioned, all the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. CHL treatment

The ganglionic blocker CHL (Tocris, Ballwin, MO, USA) was injected subcutaneously into rats (10 mg/kg body wt) 7 days prior to treatment with cholinergic agents. This concentration of CHL blocks the behavioral responses to neuroactive substances for several months (Reuben et al., 1998). It was determined previously that under these conditions CHL per se had virtually no effect on the AFC or T-cell proliferative responses in control animals (Kalra et al., 2002).

2.4. Treatment with cholinergic agents

For implantation of Alzet miniosmotic pumps (Alzet, Palo Alto, CA, USA), rats were anesthetized with an intramuscular injection of a mixture of ketamine and xylazine (87 mg and 13 mg/kg body wt, respectively). Twenty-eight-day miniosmotic pumps (Model 2ML4) were implanted subcutaneously as described previously (Geng et al., 1995). The pumps provided saline (control) and indicated concentrations of physostigmine, PB, or edrophonium. For intracerebroventricular (i.c.v.) administration, rats were anesthetized with ketamine and xylazine as described above, and stereotaxically implanted with 5-mm-long 28-gauge steel cannulae connected to a 2ML4 miniosmotic pump (Alzet Brain Infusion Kit) according to the manufacturer's instructions (Sopori et al., 1998). The coordinates were 1 mm posterior to the bregma and 1.5 mm to the midline (Pellegrino et al., 1979). Control miniosmotic pumps contained artificial cerebrospinal fluid (α-CSF) composed of 145 mM NaCl, 3.3 mM KCl, 1.3 mM $CaCl_2$, and 1.0 mM $MgCl_2$ dissolved in pyrogen-free water and filtered through a 0.2-μm membrane filter (Klir et al., 1995). The pumps were filled with α-CSF, PB (0.5 mg/kg/day), physostigmine (0.02 mg/kg/day), or edrophonium (1.5 mg/kg/day) dissolved in α-CSF. Animals were sacrificed 3 weeks after the implantation.

2.5. Immunizations

For determining the AFC response, rats were injected intravenously with (5×10^8) sheep red blood cells (SRBC) 4 days prior to sacrifice as previously described (Sopori et al., 1989).

2.6. Preparation of spleen cells

Spleen cell suspensions were prepared as described (Sopori et al., 1985). Briefly, spleens were pressed through a stainless-steel mesh, and red blood cells were lysed by treatment with a cold NH_4Cl solution. After the centrifu-

gation, cells were washed twice with cold PBS, and viable cells were counted in 0.1% eosin on a hemocytometer.

2.7. AFC assay

The primary direct AFC response was determined essentially as described by Cunningham and Szenberg (1968). Briefly, spleen cells (2×10^5) were mixed with 2% SRBC and 25 μl of guinea pig complement (Cederlane, Hornby, ON, Canada) pre-absorbed on SRBC in a final volume of 250 μl of complete medium (RPMI 1640 containing 10% fetal calf serum, 2 mM glutamine, 50 μM 2-ME, and 10 μg/ml gentamicin). Aliquots were distributed in duplicates on Cunningham slides and incubated for 45 min at 37 °C. The AFC plaques were counted and normalized to AFC/10^6 spleen cells and expressed as percent of control.

2.8. T-cell proliferation assay

The proliferative response of spleen cells to the T-cell mitogen, Concanavalin A (Con A), was measured as previously described (Sopori et al., 1987). Briefly, 2×10^5 spleen cells were cultured in 0.2 ml of the complete medium in the presence of various concentrations of Con A in microtiter plate wells. The cultures were incubated at 37 °C in the presence of 5% CO_2, and cells were harvested after 3 days by a Skatron cell harvester (Skatron, Sterling, VA, USA). T cell proliferation was assayed by adding 0.5 μCi of [^{3}H] thymidine (ICN, Irvine, CA, USA) to the culture wells 18 h before harvesting the cells.

2.9. Assay for serum corticosterone levels

Serum corticosterone levels were determined by the corticosterone RIA-kit (ICN Biochemicals, Orangeburg, NY, USA.) according to the manufacturer's instructions. As stated by the manufacturer, under these assay conditions, normal rat serum corticosterone levels vary from 50 to 400 ng/ml.

2.10. Statistical analysis

Data was analyzed for statistical significance by the Prism Software 3.0 (Graphpad, San Diego, CA, USA) using a Student's t-test. Values were considered significant at $p \leq 0.05$.

3. Results

3.1. Chronic subcutaneous administration of physostigmine, but not PB or edrophonium inhibits the AFC response

Physostigmine is a tertiary amine and crosses the BBB, while PB and edrophonium are quaternary compounds that do not ordinarily cross the BBB in appreciable quantities. However, under certain conditions such as stress and high

concentrations, they might cross the BBB (Friedman et al., 1996; Abdel-Rahman et al., 2002). To ascertain the effects of these agents on the immune system when administered peripherally at low doses, rats were treated daily via subcutaneously implanted miniosmotic pumps with saline (control), physostigmine (0.2 mg/kg/day), PB (0.5 mg/kg/day), or edrophonium (1.5 mg/kg/day) for 3 weeks. Four days prior to sacrifice, animals were injected intravenously with SRBC and the splenic AFC response was determined. Fig. 1 shows that under these conditions only physostigmine significantly suppressed the anti-SRBC AFC response, but PB and edrophonium were essentially ineffective. Raising the concentration of PB and edrophonium by approximately fivefold (i.e., 2.4 and 7.2 mg/kg/day, respectively) did not lead to a significant inhibition of the AFC (Fig. 2) or the splenic Con A-induced proliferative response (data not shown) by PB; however, edrophonium caused a small but significant decrease in the AFC response. The decrease in the AFC response by edrophonium was surprising; however, it is possible that at high concentrations small quantities of edrophonium might gain entry into the CNS, or some of the effects of edrophonium might be unrelated to its inhibition of the brain cholinesterase activity (Matteo et al., 1990). Thus, unless administered at high concentrations, peripheral administration of BBB impermeable cholinergic agents does not affect the immune system.

3.2. I.C.V. administration of PB and edrophonium inhibits AFC response

Unlike PB and edrophonium, physostigmine crosses the BBB under normal conditions. To determine whether the effects of cholinergic compounds on the immune
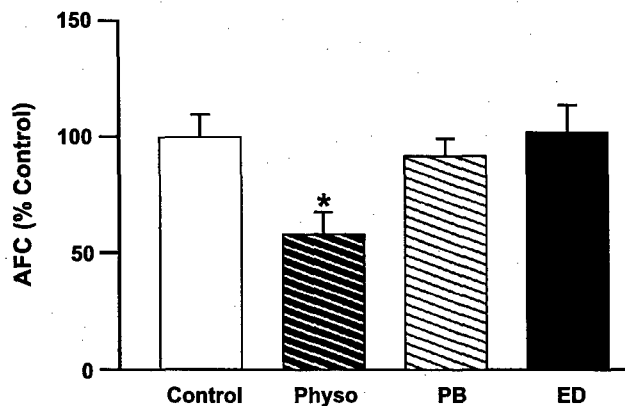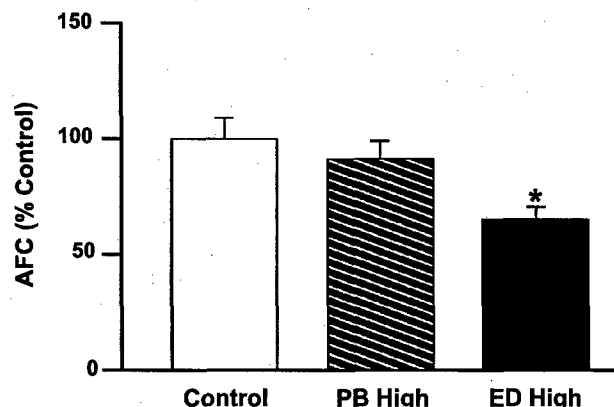

Fig. 2. Subcutaneous (s.c.) administration of high concentration of edrophonium (ED) but not of PB inhibited the AFC response. Animals (5/group) were treated subcutaneously with saline (control), PB (2.4 mg/kg/day), or edrophonium (7.2 mg/kg/day) for 3 weeks. The AFC response was determined as in Fig. 1. Differences between control and edrophonium were significant ($p = 0.011$).

system required their entrance into the CNS, we determined whether chronic treatment with low concentrations of these compounds directly into the brain (i.e., i.c.v. administration) affected the immune system. Rats were treated for 3 weeks i.c.v. with approximately 10% of the concentration of physostigmine that caused significant inhibition of the AFC response by subcutaneous route (i.e., 0.02 mg physostigmine/kg/day). For i.c.v. administration, the concentrations of PB and edrophonium used were 0.5 and 1.5 mg/kg/day, respectively. Fig. 3 shows that when given in concentrations that did not affect the AFC response through subcutaneous administration, PB and edrophonium significantly inhibited the anti-SRBC AFC response if administered via i.c.v. Moreover, even at tenfold lower concentrations, i.c.v. administration of phy-
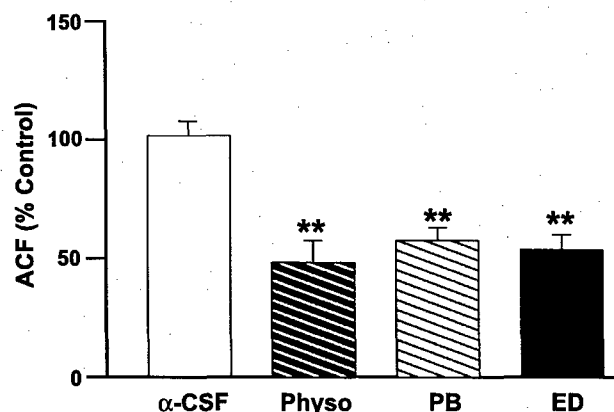

Fig. 1. Subcutaneous administration of physostigmine (physo), but not edrophonium (ED) or PB, inhibited the AFC response. Animals (N = 5/group) were treated with saline (control), physostigmine (0.2 mg/kg/day), PB (0.5 mg/kg/day) or edrophonium (1.5 mg/kg/day) via subcutaneously implanted miniosmotic pumps for 3 weeks. Four days prior to sacrifice, animals were injected intravenously with SRBC and anti-SRBC AFC response determined (see Materials and methods). The data is represented as percent of control. Statistical significance: differences between control and PB or edrophonium were statistically insignificant, whereas the difference for physostigmine was significant ($p = 0.012$).

Fig. 3. I.c.v. administration of low doses of physostigmine (Physo), PB, and edrophonium (ED) suppress the anti-SRBC AFC response. Animals (N = 5/group) were treated with α-CSF (control), physostigmine (0.02 mg/kg/day), PB (0.5 mg/kg/day) or edrophonium (1.5 mg/kg/day) for 3 weeks via i.c.v. administration. Values for α-CSF (i.c.v.) were comparable to s.c. saline control. Animals were immunized with SRBC and AFC response determined as in Fig. 1. Control values were statistically different from: physostigmine ($p = 0.003$), PB ($p = 0.001$), and edrophonium (0.001).
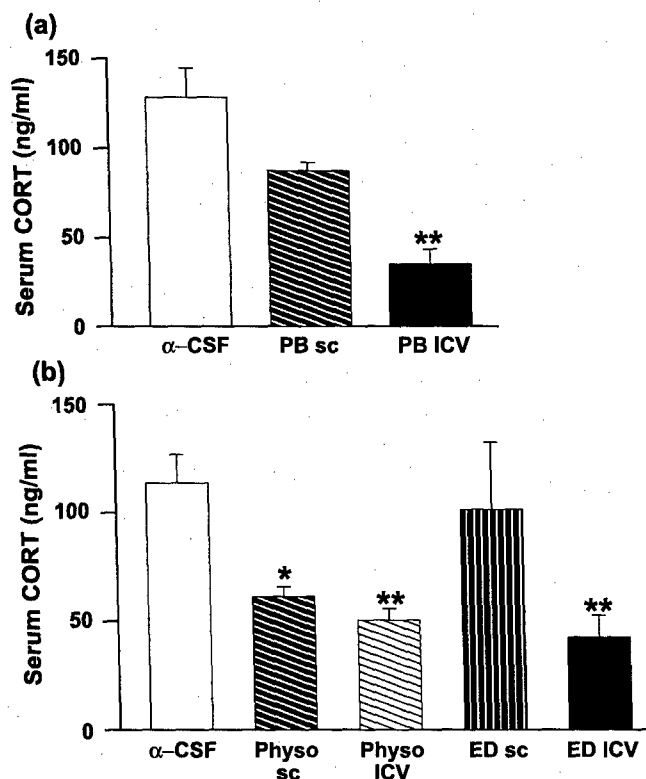
Fig. 4. I.c.v. administration of physostigmine (Physo), PB, and edrophonium (ED) suppress serum corticosterone (CORT) levels. Animals (5/ group) were treated for 3 weeks with saline or α-CSF as controls for subcutaneous (s.c.) or i.c.v. cholinergic treatments, respectively—all of the responses were similar (data not shown) and control values were given as α-CSF. (a) PB, 2.4 mg/kg/day s.c. or 0.5 mg/kg/day i.c.v.; (b) physostigmine (s.c., 0.2 mg/kg/day; i.c.v., 0.02 mg/kg/day), edrophonium (s.c., 7.2 mg/kg/day; i.c.v., 1.5 mg/kg/day). Statistical differences: PB (s.c., insignificant; i.c.v., $p = 0.002$), edrophonium (s.c., insignificant; i.c.v., $p = 0.002$), physostigmine (s.c., $p = 0.013$; i.c.v., $p = 0.005$).

sostigmine strongly inhibited the AFC response. Therefore, it is likely that the immunosuppressive effects of cholinergic agents require their entry into the CNS.

3.3. Immunosuppressive effects of cholinergic agents are not controlled through increased production of glucocorticoids

The hypothalamus–pituitary–adrenal (HPA) modulates the immune response through elevation of glucocorticoids (Turnbull and Rivier, 1999). However, sarin (a potent cholinergic agent) does not stimulate but actually strongly suppresses the serum corticosterone levels in rats (Kalra et al., 2002). To determine whether inhibition of corticosterone production is a general property of cholinergic agents, rats were treated subcutaneously or i.c.v. with PB, physostigmine, or edrophonium. Data presented in Fig. 4a shows that i.c.v. but not subcutaneous administration of low doses of PB decreased the serum corticosterone levels. Similarly, only i.c.v. administration of edrophonium decreased the serum corticosterone levels (Fig. 4b). However, physostigmine, which crosses the BBB, was effective in decreasing

the serum corticosterone level by either i.c.v. or subcutaneous administration (Fig. 4b). Thus, serum corticosterone levels are primarily modulated by the central activity of cholinergic compounds.

3.4. CHL attenuates PB-induced immunosuppression and decrease in serum corticosterone levels

To examine whether PB affected the immune system through the autonomic nervous system, animals were pretreated with CHL 7 days prior to a 3-week i.c.v. exposure to PB. Fig. 5a shows that the anti-SRBC response of PB-treated animals was significantly lower than α-CSF-treated animals, and that this decrease in the AFC response was partially moderated by treatment with CHL ($p = 0.058$). Similarly, the PB-induced drop in the serum corticosterone levels was lower in PB-treated animals that were pretreated with CHL (Fig. 5b). Combined with previously published
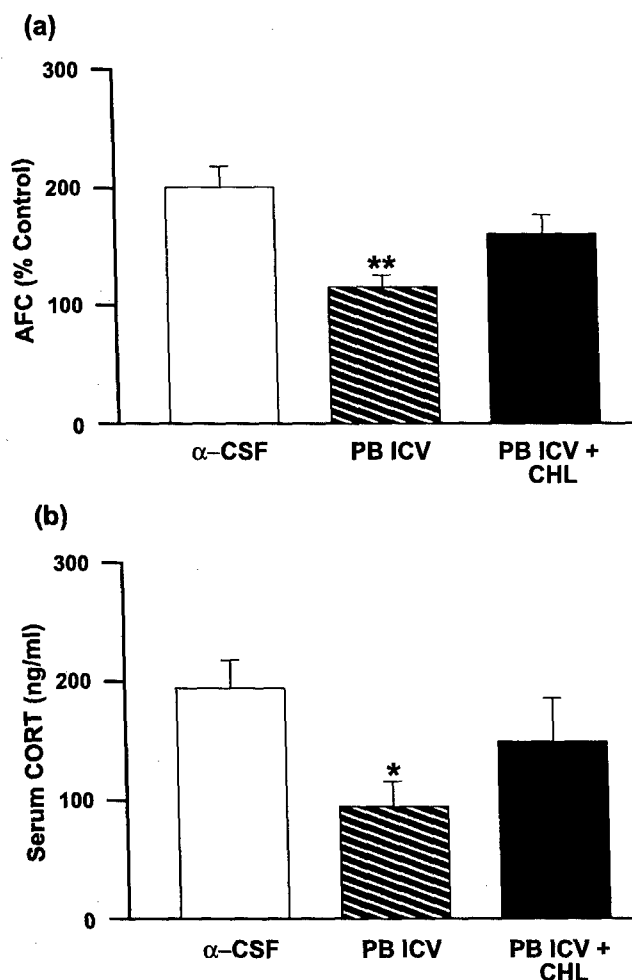

Fig. 5. CHL attenuates the inhibitory effects of i.c.v. PB administration on the AFC response (a) and serum corticosterone (CORT) levels. Animals (5/ group) were pretreated with CHL 7 days prior to 3-week treatment with α-CSF or PB (i.c.v., 0.5 mg/kg/day) as described in Materials and methods. (a) Effects on the AFC response ($p = 0.007$ between control and PB; $p = 0.058$ between PB and PB+CHL); (b) effects on CORT levels ($p = 0.027$ between control and PB).

results on sarin (Kalra et al., 2002), these data suggest that the autonomic nervous system may play an important role in mediating the immunotoxicity of cholinergic agents.

4. Discussion

Organophosphates such as pesticides and the nerve gas agent sarin irreversibly inactivate acetylcholine esterase and increase the synaptic level of acetylcholine (Grob and Harvey, 1958). Reversible cholinesterase inhibitors (such as PB, physostigmine, and edrophonium) compete with irreversible cholinesterase inhibitors and are used as therapeutics to increase the synaptic levels of acetylcholine in myasthenia gravis patients; PB also was used prophylactically for potential nerve gas exposure during the first Gulf War (Roberts et al., 1994). Several cholinergic agents have been shown to suppress the immune system; however, the mechanism of immunosuppression is not clear. Results presented herein show that, if given i.c.v., reversible cholinesterase inhibitors such as physostigmine, edrophonium, and PB suppress the immune system; thus, immunosuppression may be a common outcome of the central cholinergic toxicity.

Organophosphates are neuroactive substances, and neuroactive compounds including opiates (Nelson and Lysle, 2001), nicotine (Sopori et al., 1998; Sopori, 2002), and sarin (Kalra et al., 2002) affect the immune system through the CNS. The neuroendocrine and immune systems are intimately related during development, maturation, and the aging process (Heijnen and Kavelaars, 1999). The two systems communicate bidirectionally through hormones, cytokines, and neurotransmitters via several pathways (Blalock, 1994). The HPA axis communicates with the periphery through the release of anterior and posterior pituitary hormones that, among other effects, lead to the production of glucocorticoids from the adrenals that inhibit the immune responses (Turnbull and Rivier, 1999). The other established pathway for neuroimmune communication is the autonomic nervous system, which connects the CNS directly to visceral target tissues via the sympathetic and parasympathetic nerves (Felten and Felten, 1994; Borovikova et al., 2000). The sympathetic nerve fibers are in direct contact with T-cells in the white pulp of the spleen (Felten and Felten, 1994). To ascertain whether the immunomodulation by cholinergic agents required the CNS entry by these agents, we examined the immunosuppressive effects of the BBB permeable (physostigmine) and BBB impermeable (PB and edrophonium) cholinergic agents. Clearly, at low concentrations, all the compounds were active when given i.c.v. Subcutaneous administration of low doses of physostigmine resulted in decreased AFC response, while PB and edrophonium were essentially inactive under these conditions. Moderately high concentrations of edrophonium, but not PB, caused a small but significant reduction in the AFC response. These effects of

edrophonium may result from some of its unusual properties. For example, the duration of acetylcholine esterase inhibition by edrophonium is not tightly linked to its plasma concentrations, and it has been hypothesized that its biological effects might not totally result from its inhibition of cholinesterase activities (Blaber and Bowman, 1959; Matteo et al., 1990). Moreover, indirect evidence suggests that edrophonium may act at a site different from neostigmine and PB (Cronnelly et al., 1982). Thus, it is not clear whether the suppression of the AFC response by high doses of edrophonium results from changes in the BBB through overloading and stress (Friedman et al., 1996; Abdel-Rahman et al., 2002) or through non-central effects of high-dose toxicity. However, it is clear that, in general, the immunomodulatory effects of a cholinergic agent are dependent on its entry into the CNS.

Experiments to ascertain whether the inhibition of the AFC response by i.c.v. administration of PB, physostigmine, and edrophonium is mediated by glucocorticoids through the activation of the HPA axis indicated unequivocally that these compounds do not stimulate glucocorticoid production; in fact, these cholinergic agents are potent inhibitors of corticosterone production. These results, together with those observed with sarin (Kalra et al., 2002), clearly suggest that cholinergic agents do not suppress the immune system through increased glucocorticoid production; on the other hand decreased glucocorticoid production may be a biomarker for cholinergic toxicity.

To evaluate whether cholinergic agents affected the immune system through the autonomic nervous system, animals were pretreated with the ganglionic blocker CHL prior to exposure to PB. Results suggested that CHL treatment attenuated the suppression of the AFC response as well as that of serum corticosterone levels. However, the reversal was not complete, and might reflect incomplete inhibition of the autonomic nervous system by the dose of CHL used in these studies. Because subcutaneous administration of PB tended to decrease, although not significantly, the serum corticosterone levels without affecting the AFC response, it suggests that either the adrenal responses are more sensitive than the immune responses to cholinergic toxicity or that corticosterone production is controlled both by the central as well as peripheral activities of cholinergic agents. While additional studies are needed to sort out this ambiguity, it is clear that the autonomic nervous system plays an important role in the immunomodulatory effects of cholinergic agents.

be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation. In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985). The Lovelace Respiratory Research Institute is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

References

Abdel-Rahman, A., Shetty, A.K., Abou-Donia, M.B., 2002. Disruption of the blood–brain barrier and neuronal cell death in cingulate cortex, dentate gyrus, thalamus, and hypothalamus in a rat model of Gulf-War syndrome. Neurobiol. Dis. 10, 306–326.

Blaber, L.C., Bowman, W.C., 1959. A comparison between the effects of edrophonium and choline in skeletal muscles of the cat. Br. J. Pharmacol. 14, 456–466.

Blalock, J.E., 1994. The syntax of immune-neuroendocrine communication. Immunol. Today 15, 504–511.

Borovikova, L.V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G.I., Watkins, L.R., Wang, H., Abumrad, N., Eaton, J.W., Tracey, K.J., 2000. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405, 458–562.

Casale, G.P., Cohen, S.D., DiCapua, R.A., 1983. The effects of organophosphate-induced cholinergic stimulation on the antibody response to sheep erythrocytes in inbred mice. Toxicol. Appl. Pharmacol. 68, 198–205.

Cronnelly, R., Morris, R.B., Miller, R.D., 1982. Edrophonium: duration of action and atropine requirement in humans during halothane anesthesia. Anesthesiology 57, 261–266.

Cunningham, A.J., Szenberg, A., 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology 14, 599–600.

Desi, I., Varga, L., Farkas, I., 1978. Studies on the immunosuppressive effect of organochlorine and organophosphoric pesticides in subacute experiments. J. Hyg. Epidemiol. Microbiol. Immunol. 22, 115–122.

Felten, S.Y., Felten, D.L., 1994. Neural-immune interactions. Prog. Brain Res. 100, 157–162.

Friedman, A., Kaufer, D., Shemer, J., Hendler, I., Soreq, H., Tur-Kaspa, I., 1996. Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. Nat. Med. 2, 1382–1385.

Geng, Y., Savage, S.M., Johnson, L.J., Seagrave, J., Sopori, M.L., 1995. Effects of nicotine on the immune response: I. Chronic exposure to

nicotine impairs antigen receptor-mediated signal transduction in lymphocytes. Toxicol. Appl. Pharmacol. 135, 268–278.

Grob, D., Harvey, J.C., 1958. Effects in man of the anticholinesterase compound sarin (isopropyl methylphosphonofluoridate). J. Clin. Invest. 37, 350–368.

Heijnen, C.J., Kavelaars, A., 1999. The importance of being receptive. J. Neuroimmunol. 100, 197–202.

Kalra, R., Singh, S.P., Razani-Boroujerdi, S., Langley, R.J., Blackwell, W.B., Henderson, R.F., Sopori, M.L., 2002. Subclinical doses of the nerve gas sarin impair T cell responses through the autonomic nervous system. Toxicol. Appl. Pharmacol. 184, 82–87.

Klir, J.J., McClellan, J.L., Kozak, W., Szelenyi, Z., Wong, G.H., Kluger, M.J., 1995. Systemic but not central administration of tumor necrosis factor-alpha attenuates LPS-induced fever in rats. Am. J. Physiol. 268, R480–R486.

Matteo, R.S., Young, W.L., Ornstein, E., Schwartz, A.E., Silverberg, P.A., Diaz, J., 1990. Pharmacokinetics and pharmacodynamics of edrophonium in elderly surgical patients. Anesth. Analg. 71, 334–339.

Nelson, C.J., Lysle, D.T., 2001. Involvement of substance P and central opioid receptors in morphine modulation of the CHS response. J. Neuroimmunol. 115, 101–110.

Pellegrino, L., Pellegrino, A.S., Cushman, A.J., 1979. A Stereotaxic Atlas of the Rat Brain, 2nd ed. Plenum, New York.

Reuben, M., Louis, M., Clarke, P.B., 1998. Persistent nicotinic blockade by chlorisondamine of noradrenergic neurons in rat brain and cultured PC12 cells. Br. J. Pharmacol. 125, 1218–1227.

Roberts, D.E., Sawka, M.N., Young, A.J., Freund, B.J., 1994. Pyridostigmine bromide does not alter thermoregulation during exercise in cold air. Can. J. Physiol. Pharm. 72, 788–793.

Sopori, M., 2002. Effects of cigarette smoke on the immune system. Nat. Rev., Immunol. 2, 372–377.

Sopori, M.L., Perrone, R.S., Cherian, S., Cross, R.J., Kaplan, A.M., 1985. Immunoregulation in the rat: characteristics of a suppressor T cell that inhibits antigen-dependent cell proliferation. J. Immunol. 135, 80–86.

Sopori, M.L., Hurt, Y.L., Cherian, S., Kaplan, A.M., Diamantstein, T., 1987. Differential requirement for accessory cells in polyclonal T-cell activation. Cell. Immunol. 105, 174–186.

Sopori, M.L., Cherian, S., Chilukuri, R., Shopp, G.M., 1989. Cigarette smoke causes inhibition of the immune response to intratracheally administered antigens. Toxicol. Appl. Pharmacol. 97, 489–499.

Sopori, M.L., Kozak, W., Savage, S.M., Geng, Y., Soszynski, D., Kluger, M.J., Perryman, E.K., Snow, G.E., 1998. Effect of nicotine on the immune system: possible regulation of immune responses by central and peripheral mechanisms. Psychoneuroendocrinology 23, 189–204.

Street, J.C., Sharma, R.P., 1975. Alteration of induced cellular and humoral immune responses by pesticides and chemicals of environmental concern: quantitative studies of immunosuppression by DDT, aroclor 1254, carbaryl, carbofuran, and methylparathion. Toxicol. Appl. Pharmacol. 32, 587–602.

Turnbull, A.V., Rivier, C.L., 1999. Regulation of the hypothalamic–pituitary–adrenal axis by cytokines: actions and mechanisms of action. Physiol. Rev. 79, 1–71.